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Solution conformation of gramicidin S: An intramolecular nuclear Overhauser effect study

(proton NMR/antibiotic/peptide/protein)

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ABSTRACT **The solution conformation of gramicidin S in deuterated dimethyl sulfoxide was investigated by using the intramolecular nuclear Overhauser effect experiment. Experimental Overhauser enhancements were compared with predicted values for each of the nine most-stable conformations (M1–M9) calculated by Dygert *et al.* on the basis of energy-minimization procedures [Dygert, M., Gō, N. & Scheraga, H. A. (1975) *Macromolecules* 8, 750–761]. By using statistical hypothesis testing, the three lowest-energy conformations (M1, M2, and M3) were shown to give the best fit with the experimental data. All other conformations (M4–M9) were found to be inconsistent with the experimental data.**

Intramolecular nuclear Overhauser effects (NOEs) are very sensitive to interproton distances when dipole-dipole interaction is the predominant mechanism of proton relaxation (1). Because of this distance dependence, NOE measurements have been used extensively in conformational studies of small molecules (1); however, their application to peptides is relatively recent (2–7).

The cyclic decapeptide antibiotic gramicidin S has been the subject of many experimental [ref. 8 (also see various refs. cited in this review); 9–12] and theoretical (13) investigations. Recently, Rae *et al.* (4) and Jones *et al.* (3) estimated some interproton distances of gramicidin S from NOE data, vicinal coupling constants, and relaxation time measurements and obtained results consistent with the distances of the low-energy structure calculated by Dygert *et al.* (13). However, because each interproton distance can correspond to at least two torsional angles (ϕ or ψ for the backbone and χ for the side chains), other higher-energy conformations were not rigorously excluded. In this paper, we make a detailed comparison between the experimental NOE data for gramicidin S in deuterated dimethyl sulfoxide $(^2H_3C)_2SO$ at 400 MHz and the NOE values predicted for each of the nine low-energy structures computed for this decapeptide by Dygert *et al.* on the basis of potential energy calculations. The method of analysis used in this work is similar to that used by Krishna *et al.* (2) for valinomycin-K^+. Such a detailed comparison formed the basis for excluding structures that are inconsistent with the experimental data.

MATERIALS AND METHODS

Gramicidin S (2.5 mg) (Sigma) was dissolved in 0.5 ml of 100% $(^2H_3C)_2SO$ (Merck), and the sample was transferred to a 5-mm outside-diameter NMR sample tube (Wilmad Glass, Buena, NJ) for the experiments. NOE experiments were performed in the pulsed Fourier transform mode on a Bruker WH-400 spectrometer equipped with an Aspect 2000 computer. The probe temperature was 297 ± 1 K. In the NOE experiment, the resonance signal of interest was selectively presaturated for 4 sec before the observation pulse. The decoupler power was set such that the irradiation was highly selective; the decoupler power spillover to the neighboring resonance signals was less than 2% in general, as determined from the difference spectra. For the control spectrum, the irradiation frequency was set away from the spectral region by more than 2 kHz. The degree of saturation of the irradiated lines was 80–90%, and the observed NOEs were finally corrected for 100% saturation of the irradiated peak by dividing by the degree of saturation. One thousand scans were accumulated for each spectrum. To minimize the effects associated with spectrometer drift and to obtain clean difference spectra, the accumulation was alternated every eight scans between the control and the NOE experiments. A 0.5-Hz exponential line broadening was used in Fourier transforming the free induction decays. The NOE difference spectra were obtained by subtracting the control spectrum from the double resonance spectra. The NOE enhancements were calculated as fractional intensity changes for each resonance signal. For overlapping signals and for the $C^\beta H_2$ multiplet of D-phenylalanine, integrated intensities were used in computing the NOEs.

RESULTS AND DISCUSSION

The nuclear Overhauser effect experiment measures the steady-state effect of saturation of a specific spin transition or group of transitions on the intensities of other resonances in the NMR spectrum. The fractional change in the intensity of spin i when a spin s (or a group of such spins) is saturated can be computed by solving a set of simultaneous equations of the form (2)

$$\sigma_{ii} f_i(s) + \sum_j \sigma_{ij} f_j(s) = \sum_s \sigma_{is}, \quad j \neq i,s \qquad [1]$$

where the summation on the right-hand side involves only the saturated spins. The σ_{ii} and σ_{ij} terms are functions of interproton distances, the molecular rotational correlation time, the Larmor frequency, and the proton magnetogyric ratio. In addition, for amide hydrogens, the σ_{ii} term also takes into account the dipolar interaction between the proton and the directly bonded ^{14}N nucleus (2). In our analysis, we have assumed that the rotational diffusion of gramicidin S is isotropic. For a given correlation time and resonance frequency and a given set of interproton distances calculated from the model, the theoretical NOE values can be calculated from Eq. 1. The 2-fold symmetry of the molecule has also been taken into account. A typical

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Abbreviation: NOE, nuclear Overhauser effect.

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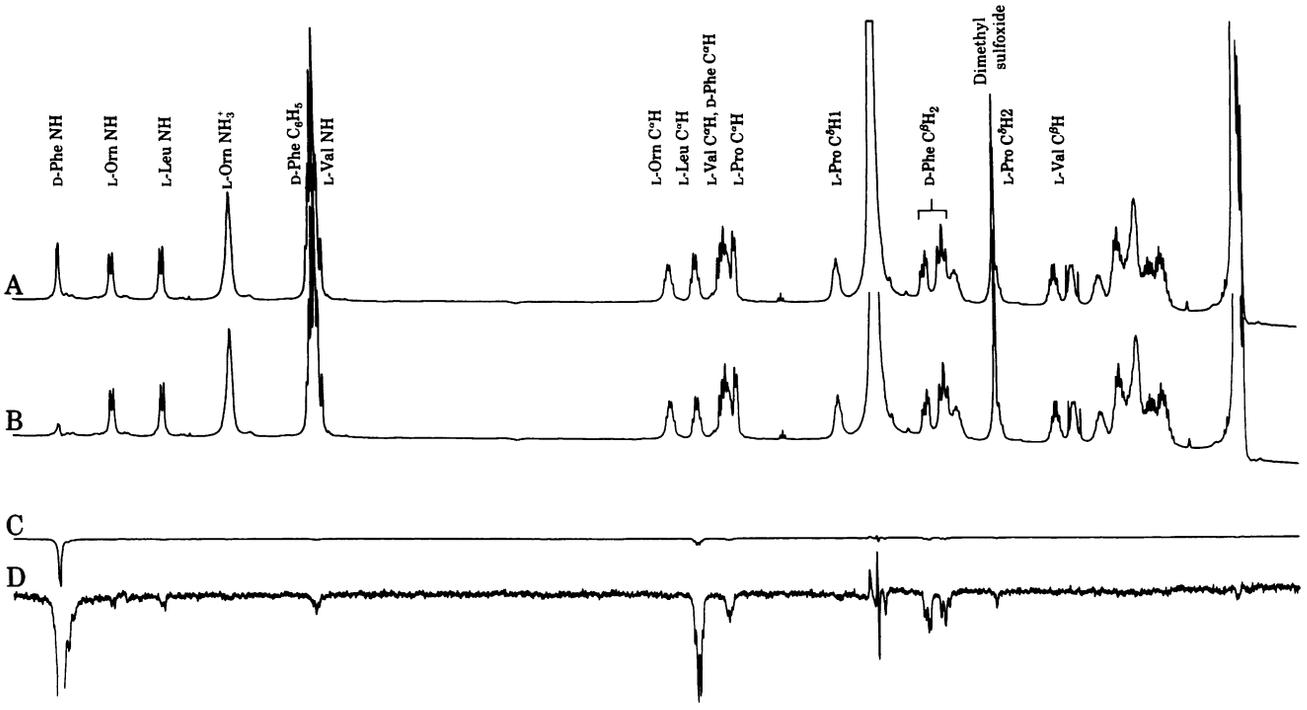

FIG. 1. Typical intramolecular ^1H NOE experiment at 400 MHz on gramicidin S in $(^2H_3C)_2SO$. (*A*) Control. (*B*) Double resonance spectrum obtained by steady-state saturation of D-phenylalanine NH. (*C*) Difference spectrum (*B* − *A*). (*D*) Amplified (16-fold) difference spectrum.

steady-state NOE experiment on gramicidin S is shown in Fig. 1. The assignments of the various resonances are those of Jones *et al.* (3). The increased chemical shift dispersion available at 400 MHz enabled us to measure a large number (46) of nonzero NOEs (Table 1). In view of this large number of nonzero NOEs, we have not included several zero NOEs in the analysis, even though, in principle, they may be treated as additional data. It may be seen that, even though the NOEs are negative at 400 MHz, we observe highly differential effects. This implies, in turn, that spin diffusion effects are not dominant under the operating conditions (i.e., the operating frequency and the rotational correlation time of the molecule) and that the steady-state NOE experiment should be useful in unraveling the conformational details of gramicidin S. In the spin diffusion limit, it is more advantageous to perform transient NOE experiments involving either selective progressive saturation (2, 14) or selective inversion (2, 15).

The gramicidin S structures were generated by using the empirical conformational energy program for peptides (16) with standard bond angles, bond lengths, and the reported torsional angles (ϕ, ψ, and χ) for the M1–M9 conformations (13). All the computations were performed on an IBM 370 computer. Theoretical NOEs were generated by solving the set of simultaneous equations (Eq. 1). For each model, the correlation time, τ_c, was optimized in a least-squares sense with respect to the experimental data, so that the goodness-of-fit parameter, χ^2, was minimum, and the agreement factor, R (17, 18), was calculated (Table 2). The R values varied a great deal from one model to another. However, the relatively low values for the M1, M2, and M3 structures suggest better agreement for these three models. Whether the differences in the R values are statistically significant can be judged by using Hamilton's R-factor-ratio criterion (17, 18). This test provides a basis for rejecting alternative models as inconsistent with the experimental data in comparison with models that give rise to lower R values (see Table 2). Statistical hypothesis testing involving Hamilton's R-factor-ratio criterion has been applied successfully in x-ray crystallography (17) and in NMR studies (2, 19–23). Because, in analyzing the 46 NOEs, only one parameter (τ_c) was optimized, the number of degrees of freedom equals 45. Each theoretical model is completely defined by 23 torsional angles because of the 2-fold symmetry (5 ϕs, 5ψs, and 13 χs). As a conservative estimate, the dimension of the hypothesis has been taken as 24.‖ From tables of significant points for the R-factor ratio (17, 18), we can compute the confidence level for rejecting the alternative model with respect to the M3 model (see Table 2). It is seen that conformations M4–M9 can all be rejected as inconsistent with the experimental data with greater than 99.5% confidence. On the other hand, the two lowest-energy structures (M1 and M2) could not be rejected in comparison with the M3 model, even at the 0.5 significance level. We thus conclude that the antiparallel β-pleated sheet type structures M1–M3 are equally successful on a statistical basis, in explaining the NOE data. The remaining models (M4–M9) are all inconsistent. The optimized rotational correlation times obtained in this study are in good agreement with those obtained by Jones *et al.* by using a different procedure (24).

In addition to the M1–M9 conformations, we have also examined the X1, X2, and X3 structures given in table VII of ref. 13. The X1 and X2 structures are derived from the models given by De Santis and Liquori (25), and the X3 structure is derived from the model of Ovchinnikov *et al.* (26). Examination of the R factors** for these structures shows that these are as successful as the M1–M3 structures in explaining the NOE data.

‖ Here we do not take into account the side-chain angles for proline [which is assumed to be in the *trans* form (13)] and the various ωs(≡180°), because these are invariant for the various models. Even though the ϕ angle (−75°) for the proline residue is the same for all the structures, we include this to be on the conservative side.

** We obtained R values of 0.177, 0.166, and 0.174 for the X1–X3 structures, respectively. The optimized correlation times are 11.24, 11.43, and 11.25 (in units of 10^{-10} sec), respectively.

Table 1. Comparison between experimental proton NOE enhancements (f_{io}s) for gramicidin S in $(^2H_3C)_2SO$ and theoretical values (f_{ic}s)

Spin			f_{ic}		Spin			f_{ic}	
Saturated	Observed	f_{io}*	M3	M4	Saturated	Observed	f_{io}	M3	M4
Phe NH	Leu NH	−0.021	−0.013	−0.145	Pro $C^{\alpha}H$	Orn NH	−0.024	−0.002	−0.001
	Leu $C^{\alpha}H$	−0.165	−0.193	−0.035		Phe C_6H_5/Val NH	−0.016	−0.004	−0.004
	Val/Phe $C^{\alpha}H$	−0.021	−0.030	−0.025		Leu $C^{\alpha}H$	−0.029	−0.001	−0.0
	Phe $C^{\beta}H_2$	−0.058	−0.080	−0.029		Pro $C^{\delta}H1$	−0.042	−0.009	−0.007
						Phe $C^{\beta}H_2$	−0.013	−0.004	−0.001
Orn NH	Phe C_6H_5/Val NH	−0.005	−0.002	−0.002		Pro $C^{\delta}H2$	−0.039	−0.009	−0.008
	Orn $C^{\alpha}H$	−0.043	−0.048	−0.043		Val $C^{\beta}H$	−0.013	−0.002	−0.001
	Leu $C^{\alpha}H$	−0.014	−0.004	−0.005					
	Val/Phe $C^{\alpha}H$	−0.141	−0.111	−0.070	Pro $C^{\delta}H1$	Phe C_6H_5/Val NH	−0.029	−0.048	−0.029
	Val $C^{\beta}H$	−0.056	−0.014	−0.056		Pro $C^{\delta}H2$	−0.367	−0.363	−0.331
Leu NH	Phe NH	−0.019	−0.015	−0.163	Pro $C^{\delta}H2$	Phe NH	−0.019	−0.020	−0.021
	Phe C_6H_5/Val NH	−0.006	−0.002	−0.004		Phe C_6H_5/Val NH	−0.039	−0.047	−0.027
	Orn $C^{\alpha}H$	−0.177	−0.175	−0.140		Pro $C^{\delta}H1$	−0.344	−0.355	−0.331
	Leu $C^{\alpha}H$	−0.047	−0.060	−0.082					
					Val $C^{\beta}H$	Orn NH	−0.050	−0.017	−0.071
Orn $C^{\alpha}H$	Phe NH	−0.008	−0.006	−0.025		Phe C_6H_5/Val NH	−0.013	−0.019	−0.004
	Orn NH	−0.036	−0.052	−0.039		Val/Phe $C^{\alpha}H$	−0.033	−0.040	−0.081
	Leu NH	−0.176	−0.178	−0.095		Pro $C^{\alpha}H$	−0.034	−0.006	−0.004
	Val/Phe $C^{\alpha}H$	−0.015	−0.010	−0.007					
					Val/Phe $C^{\alpha}H$	Phe NH	−0.063	−0.049	−0.040
Leu $C^{\alpha}H$	Phe NH	−0.144	−0.146	−0.016		Orn NH	−0.175	−0.148	−0.088
	Orn NH	−0.006	−0.002	−0.003		Leu NH	−0.017	−0.006	−0.010
	Leu NH	−0.042	−0.039	−0.033		Phe C_6H_5/Val NH	−0.030	−0.053	−0.059
	Phe $C^{\beta}H_2$	−0.016	−0.014	−0.003		Orn $C^{\alpha}H$	−0.037	−0.012	−0.010
						Pro $C^{\delta}H1$	−0.145	−0.113	−0.075
						Phe $C^{\beta}H_2$	−0.044	−0.069	−0.056
						Pro $C^{\delta}H2$	−0.117	−0.106	−0.147
						Val $C^{\beta}H$	−0.042	−0.045	−0.080

* Experimental error, ±0.01.

In this investigation, we have examined each of the theoretical conformations separately under the assumption that a single correlation time describes both the backbone and the various side chains. This is an oversimplification for interproton vectors that involve the side-chain protons because the latter undergo internal motions in addition to the overall reorientation in solution. A more rigorous analysis of the NOE data should take into consideration the motion of the side-chain protons among various rotamers. The possibility exists that our experimental data might be better explained by a conformational equilibrium of gramicidin S among some backbone and the various side-chain orientations. Such a possibility is also suggested by a recent investigation of the vicinal coupling data on gramicidin S (27, 28). In Table 1, the experimental NOEs are compared with the values predicted for the M3 and M4 structures by using the correlation times given in Table 2. It may be seen from this table that, even though several of the predicted NOEs are different for these two structures, a significant number are approximately equal. This observation suggests that caution should be exercised in drawing conclusions based on a few isolated NOE experiments or comparison between the experimental data and predictions for a single conformation. Our study shows the advantage of a total approach in which a large number of NOEs are simultaneously compared, by using statistical criteria, with

Table 2. Analysis of intramolecular NOE enhancements of gramicidin S in $(^2H_3C)_2SO$

	M1	M2	M3	M4	M5	M6	M7	M8	M9
χ^{2}*	3.66	3.66	3.29	24.77	23.28	12.54	22.30	8.19	18.36
R†	0.184	0.184	0.174	0.478	0.464	0.340	0.454	0.275	0.412
R ratio‡	1.058	1.058	1	2.747	2.667	1.954	2.609	1.581	2.368
Confidence level, %	—	—	—	99.5	99.5	99.5	99.5	99.5	99.5
τ_c§	11.24	11.25	11.98	11.09	11.24	12.79	10.70	12.32	12.10

* $\chi^2 = \sum_i [(f_{io} - f_{ic})/\sigma_i)^2]/(N_x - N_p)$; the values reported are the minimum values in the fit. N_x, total number of NOE measurements included in the fit. N_p, number of parameters used in the optimization. σ_i is the standard deviation and has been assumed to be 0.01 for all measurements.

† $R = \left[\sum_i (f_{io} - f_{ic})^2 / \sum_i f_{io}^2\right]^{1/2}$

‡ With respect to the lowest value (0.174, for the M3 conformation).

§ Correlation time (in units of 10^{-10} sec) that corresponds to the best agreement between experiment and theory (i.e., minimum χ^2 value). Estimated error in the fitted τ_c values is ±0.15.

those predicted by different molecular models. This method could be further improved by including vicinal coupling-constant data in the optimization with respect to each model. The method of analysis considered here is applicable not only to various peptides but also to proteins and nucleic acids.

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